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Distribution of *Gonipterus* Species and Their Egg Parasitoids in Australia: Implications for Biological Control

Michelle L. Schröder [1,*], Helen F. Nahrung [2], Natalia M. de Souza [2], Simon A. Lawson [2], Bernard Slippers [3], Michael J. Wingfield [3] and Brett P. Hurley [1]

1 Department of Zoology and Entomology, Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria 0028, South Africa; brett.hurley@fabi.up.ac.za

2 Forest Research Institute, University of the Sunshine Coast, Sippy Downs, QLD 4558, Australia; hnahrung@usc.edu.au (H.F.N.); ndesouza@usc.edu.au (N.M.d.S.); slawson@usc.edu.au (S.A.L.)

3 Department of Biochemistry, Genetics and Microbiology, Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria 0028, South Africa; bernard.slippers@fabi.up.ac.za (B.S.); mike.wingfield@fabi.up.ac.za (M.J.W.)

* Correspondence: michelle.schroder@fabi.up.ac.za; Tel.: +27-420-3937

Abstract: *Gonipterus* species are pests of *Eucalyptus* plantations worldwide. The egg parasitoid wasp *Anaphes nitens* is used in many countries for the biological control of *Gonipterus* spp. Recent taxonomic studies have shown that the three invasive *Gonipterus* spp., which were previously considered as *G. scutellatus*, form part of a cryptic species complex. These taxonomic changes have implications for the biological control of *Gonipterus* spp. The aims of this study were to understand the species composition and distribution of *Gonipterus* spp. and their egg parasitoids in Australia. *Gonipterus* spp. adults and egg capsules were collected in south-eastern Australia and Tasmania. Adult *Gonipterus* were identified using morphology and DNA barcoding. Parasitoids were reared from *Gonipterus* egg capsules and identified. Thirteen *Gonipterus* species were collected: twelve species were found on the Australian mainland and one species in Tasmania. These included three described species, four previously recognized but undescribed species, two undescribed species and four unidentified species. Five egg parasitoid species that attack *Gonipterus* spp. were identified. *Anaphes nitens*, *Centrodora damoni* and *Euderus* sp. were identified on the Australian mainland and *A. tasmaniae* and *A. inexpectatus* were identified in Tasmania. The results from this study will contribute to the improvement of *Gonipterus* biological control in the future.

Keywords: *Anaphes nitens*; *Centrodora damoni*; *Euderus* sp.; *Eucalyptus* pest; *Gonipterus scutellatus* species complex

1. Introduction

A number of strategies and protocols underpin the efficiency and success of biological control programs [1]. These include studies from the native ranges of the target pests aimed at understanding the diversity and ecology of natural enemies within the native range [2], searching for natural enemies in a region that is climatically similar to that of the introduced range [2,3] and collecting natural enemies from native populations of the pest that are genetically similar to those of the invasive population [2,4,5]. All of these approaches depend on a thorough understanding of the taxonomy, distribution and population structure of the target pest and its natural enemies in their native ranges [6–11].

The existence of cryptic species complexes can hamper the successful implementation of biological control. If the pest or biological control agent is part of a cryptic species complex, the misidentification of either of these components poses a significant risk. This can lead to the introduction of mismatched species of biological control agents, which in turn can lead to failure or inefficient control [12–16]. Combining ecological, behavioural and biosystematic studies with the morphological descriptions of species can contribute to

detecting cryptic species [17–22]. For example, two cryptic species of *Ganaspis brasiliensis* (Ihering) (Hymenoptera: Figitidae), a candidate biological control agent for *Drosophila suzukii* Matsumura (Diptera: Drosophilidae) displayed different host specificity through different habitat preferences. *Drosophila suzukii* only attacks ripening fruit in its invaded range. One genetically distinct group of *G. brasiliensis* was found to only attack larvae in ripening fruit, whereas the other group attacked *Drosophila* larvae irrespectively of their food source. The *G. brasiliensis* group that is more habitat specific is thus the preferred candidate for biological control [23]. It is therefore important to determine whether cryptic species are present at the start of biological control programmes, and if this is the case, investigate the potential implication of this cryptic diversity on the program.

Gonipterus spp. Schöenherr (Coleoptera: Curculionidae) native to Australia, are defoliators of *Eucalyptus* spp. Female beetles oviposit on new leaves in clusters of up to 20 eggs that they cover with an excrement. The emerging larvae feed on the epidermis of the new leaves, resulting in crown defoliation and significant yield loss. Adult beetles are also leaf feeding but the larvae cause most of the damage [24]. Merchantable wood loss has been estimated to be between 29 and 86% with defoliation levels from 50 to 100% [25].

Invasive populations of *Gonipterus* were thought to be a single species (*G. scutellatus*) but are now recognised as three species in a cryptic complex of four described and four undescribed species in Australia [14]. The invasive species are *G. platensis* Marelli, *G. pulverulentus* Lea and *Gonipterus* sp. n. 2. *Gonipterus* sp. n. 2 is native to eastern mainland Australia and is invasive in Western Australia, Tasmania, Africa and parts of Europe [14,26]. *G. pulverulentus* is endemic in New South Wales, Australian Capital Territory and Tasmania and is invasive in South America. *G. platensis* is native to Tasmania, and invasive in North and South America, parts of Europe and Australasia [14].

A single parasitoid species, *Anaphes nitens* (Girault) (Hymenoptera: Mymaridae) was introduced to control the invasive *Gonipterus* spp. [24,27,28], based on the premise that it was one globally invasive pest species. *Anaphes nitens* was originally detected in South Australia, Victoria and New South Wales, but material for the initial shipment was only collected in South Australia due to high numbers in this region [24]. The first introductions were made in South Africa (1926) and New Zealand (1927, 1929) [24,29]. This classical biological control program was successful, and by 1950, *Gonipterus* populations in South Africa were considered to be under economic control [24]. Following the success of the programme in South Africa, *A. nitens* was sourced from this country and introduced from there to countries in the Americas, Europe and Africa where *Gonipterus* spp. had become invasive [27,30–33].

Despite the initial success of the *Gonipterus* biological control programmes using *A. nitens*, pest resurgence has been observed in the invaded range of the pest [24,25,34–36]. The taxonomic confusion regarding the identity of the target pest species, as well as abiotic factors have been identified as possible contributing factors to the observed population outbreaks [14,24,25,37]. The discovery of cryptic diversity in *Gonipterus* has thus been an important driver for further studies in the native range to improve the biological control of *Gonipterus*.

It is not clear how cryptic diversity has globally affected the classical biological control programme for *Gonipterus* spp. Historical distribution records are not reliable due to the incorrect identification of *Gonipterus* spp. and distribution records of *Gonipterus* spp. are limited to two studies of which only one includes the Australian mainland [14,26]. In addition, limited information is available regarding the distribution of the egg parasitoids within the native range.

Recent surveys and identification of the natural enemies that attack *Gonipterus* spp. in Tasmania were undertaken, where *G. platensis* and *G. pulverulentus* occur [26,38]. Only historical records of the natural enemies are available from the Australian mainland where *Gonipterus* sp. n. 2 is native. In this study, the known distribution range of *Gonipterus* species in Australia was surveyed to gain a wider perspective of *Gonipterus* species composition, geographical distribution, host–plant relationships and egg parasitoids within its native

and introduced range within Australia. This is especially in light of recent taxonomic understanding of the pest. The relevance of these results to biological control programmes of *Gonipterus* was also considered.

2. Materials and Methods

2.1. Insect and Plant Collections

Gonipterus adults and egg capsules were collected from mature and juvenile *Eucalyptus* trees in plantations, recreational parks and roadsides in South Australia (SA), Victoria (VIC), New South Wales (NSW), Australian Capital Territory (ACT), Queensland (QLD), Western Australia (WA) and Tasmania (TAS) (Figure 1). A total of 373 locations were inspected for the presence of *Gonipterus*. The sampling locations were selected by driving and stopping at regular intervals along the road, inspecting trees at recreational parks and plantation trees where possible. From these sampling points, *Gonipterus* was collected from 109 locations. Two collecting trips were done in early and late summer of 2017 in SA, VIC, NSW and ACT. Collections from TAS comprised only one sampling occasion, in early summer of 2017, but was deliberately limited because of recent surveys in that state [26,38]. Collections in QLD were done in late summer in 2017 and additional ad hoc sampling was done in 2018 and 2019. Collections in WA were done in 2018 from plantations. All collection areas other than WA include the known native distribution of the *G. scutellatus* species complex in Australia. Leaves and seed capsules were collected from mature *Eucalyptus* spp. from which beetles were collected and submitted to the Queensland Herbarium at the Brisbane Botanic Gardens, Mt. Coot-tha for identification. Collections were not limited to mature trees and included juvenile trees. No distinction was made between planted and native trees within the respective regions. GPS location data were recorded for each collection point.

(a)   (b)
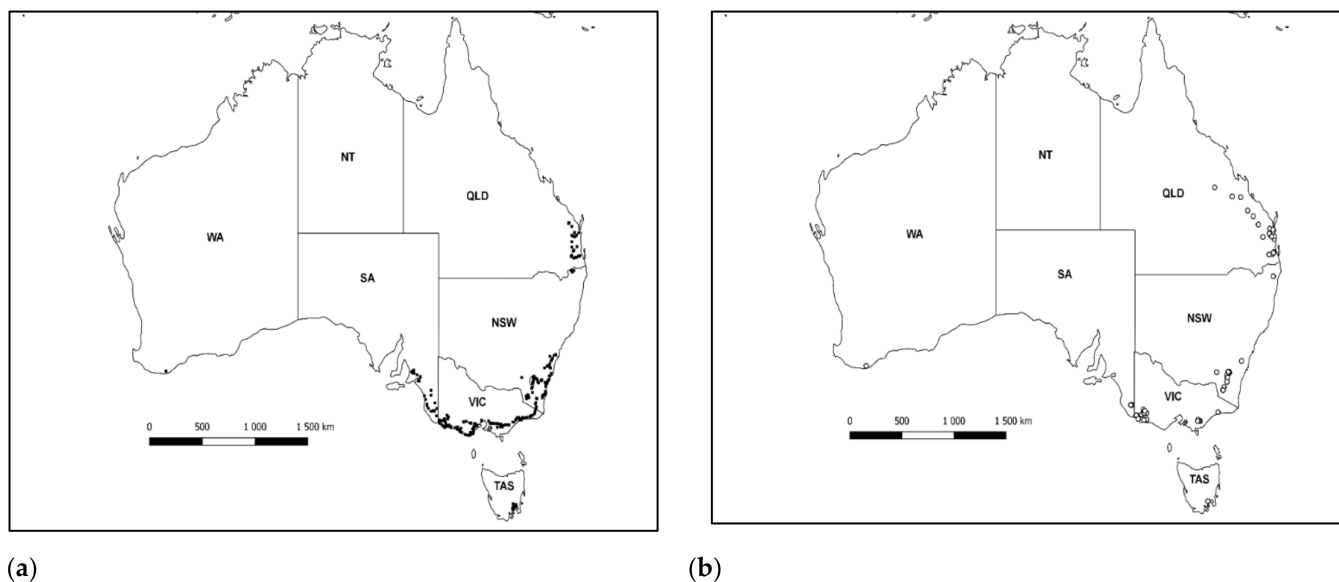
Figure 1. Geographical representation of the collection points where searches were made for the presence of *Gonipterus* species and their parasitoids: (**a**) collection points where searches were made for *Gonipterus* but not found; and (**b**) where *Gonipterus* was found.

Adult beetles were killed by freezing and were stored in 99% ethanol prior to identification. Egg capsules were individually placed into gelatine capsules or 0.5 mL tubes in an incubator at 20 °C with 70% humidity. All parasitoids that emerged from the egg capsules were collected for identification.

2.2. Species Identification

Adult *Gonipterus* were identified using morphological characteristics and sequences of the Cytochrome Oxidase 1 (CO1) gene in the mitochondrial DNA. The most reliable morphological characteristic for the species identification for *Gonipterus* is the morphology of the male genitalia [14]. Therefore, only the males were identified by means of morphology (292 males). The females were identified by DNA barcoding. Between one and five females per collection site were used for identification. Sequence data from the DNA barcoding region were also included from identified males. Sequences from GenBank were used as reference samples.

Morphological identification of adult male *Gonipterus* individuals was done by incubating the male abdomens in a warm 10% solution of potassium hydroxide (KOH). The aedeagus was removed from the KOH solution, rinsed and dissected in 70% ethanol and examined in glycerine. The remains of the abdomen and the aedeagus were placed in genital vials with glycerine for temporary storage. Identifications of *Gonipterus* spp. were confirmed by R. Oberprieler and representative specimens were deposited with the Australian National Insect Collection (ANIC), CSIRO, Black Mountain, ACT.

Adult parasitoid wasps were placed in ethanol in a Petri dish and identified using a dissecting microscope (ZEIS) with 100× magnification and the keys by Huber and Prinsloo [39] and Prinsloo (Pers. comm.). Representative samples of each species were sent to G. Prinsloo (formerly affiliated with the Agricultural Research Council (ARC)-Biosystematics, Pretoria, Gauteng, South Africa) for species confirmation.

2.3. DNA Barcoding

DNA extraction. A total of 108 *Gonipterus* adults (17 male, 80 female and 11 unknown) were used for DNA barcoding. DNA was extracted from the wing muscles or hind leg of adult insects. A ZyGEM PrepGEM® Insect DNA extraction kit and manufacturer's protocol were used. DNA extractions were diluted to approximately 50 ng/μL and stored at −20 °C.

Polymerase chain reaction (PCR). A 331 bp fragment of the CO1 gene region of the mitochondrial DNA was amplified using previously published primers (Table 1). PCR was performed for each sample using ProFlex PCR system (ThermoFischer Scientific Inc., Johannesburg, South Africa). Each 25 μL reaction contained 2.5 μL 10× PCR buffer (ROCHE, Roche Diagnostocs, Midrand, South Africa) 3 μL of 25 mM magnesium chloride (ROCHE), 0.5 μL Faststart Taq Polymerase (ROCHE), 2.5 μL DNTPs (ROCHE) (10 μM of each DNTP), 1 μL of each forward and reverse primer (Whitehead Scientific (Pty) Ltd. Johannesburg, South Africa; Inqaba Biotec ™, Pretoria, South Africa) diluted to 10 mM, 13 μL of distilled water and 1 μL of template DNA. The PCR thermal cycle program included a denaturation period of 5 min at 96 °C, 40 cycles of 1 min at 94 °C, 1 min at annealing temperature and 1 min and 30 s at 72 °C, followed by a final extension period of 10 min at 72 °C.

Table 1. Primers used for PCR amplification and sequencing of the *Gonipterus* species.

Primer Name	Direction	Region	Location of 3′ End	Reference	Sequence (5′-3′)
C1-J-2183	F	CO1	2183	Simon et al., 1994	CAA CAT TTA TTT TGA TTT TTT GG
C1-N-2659c	R	CO1	2659	Laffin et al., 2005	ACT AAT CCT GTG AAT AAA GG
TL2-N-3014	R	CO1	3014	Simon et al., 1994	TCC AAT GCA CTA ATC TGC CAT ATT A
Ron	F	CO1	1751	Simon et al., 1994	GGA TCA CCT GAT ATA GCA TTC CC
K698	F	CO1	1460	Simon et al., 1994	TAC AAT TTA TCG CCT AAA CTT CAG CC
K741 1999	R	CO1	2578	Caterino and Sperling 1999	TGG AAA TGT GCA ACT ACA TAA TA
Gon-F	F	CO1	2215	Mapondera et al., 2012	GGA GTA CTC GGG ATA ATT TAC G
G118RT	F	CO1	2120	This study	GGA GGG GGT GAC CCT ATT T
G118RT	R	CO1	2778	This study	AGT CCG AGT ATC GAC GAG GT

Note: Primers were used in the following pairs: (1) C1-J-2183 and C1-N-2659c; (2) C1-J-2183 and TL2N-N-3014; (3) Ron and K741 1999; (4) K698 and K741 1999; (5) GON-F and TL2-N-3014; and (6) G118RT F and G118RT R.

PCR products were visualized on a 1% agarose gel using electrophoresis (BioRad Gel DocTM Ez Imager and the software Image Lab v 4.0 build 16). PCR products were purified using the QIAquickTM PCR Purification Kit (QIAGEN) following the manufacturer's instructions and visualised on a 1% agarose gel as described above. Purified PCR products were used for sequencing. Each 10 mL sequence cycle reaction consisted of 2 μL sequencing buffer (Applied Biosystems[TM], Thermo Fischer Scientific, Pretoria, South Africa), 0.5 μL BigDye (Applied Biosystems[TM]), 0.5 μL primer for each forward and reverse primer separately, 6 μL distilled water and 2 μL purified PCR product. The thermal cycle reaction program included an initial denaturation of 2 min at 96 °C, 35 cycles of 30 s at 96 °C, 15 s at annealing temperature, and 4 min at 60 °C. Cycle sequencing products were cleaned using the Ethanol/NaAC precipitation of BigDye Terminator v3.1 DNA sequencing reactions protocol from the ABI manual. Samples were sequenced using an ABI PrismTM 3100 Genetic Analyzer (Applied Biosystems[TM]).

2.4. Data Analysis

Phylogenetic analysis. Forward and reverse sequences were manually checked for base accuracy and trimmed using Chromas 2.6.2 [40]. Sequence contigs were created using BioEdit Sequence alignment editor version 7.2.5 [41] and aligned using ClustalW in MEGA 6 [42]. DNA sequences were translated to amino acids and searched for the presence of stop codons using AliView version 1.22 [43]. Seventy-three sequences of *Gonipterus* from Genbank (FJ88529.1-FJ88529.1, JN391478.1-JN391486) were added to the dataset before analysis. CO1 sequences (obtained from GenBank) for the closely related genus *Haplonyx* sp. were used as an outgroup. A neighbour joining tree using Maximum Composite Likelihood Model and 1000 bootstrap replicates was calculated using MEGA 6.

Species distribution: GPS and species identification data were used to map the geographical distribution of *Gonipterus* species and their egg parasitoids in Australia. Distribution maps were generated using GIS software (QGIS version 3.2.2, 2018).

3. Results

3.1. Identification and Distribution of Gonipterus Species

Thirteen *Gonipterus* spp. were identified based on the genitalia sclerites of 292 adult males (Figure 2). Five species, *Gonipterus* sp. n. 1–4 and *G. pulverulentus* are recognised members of the *G. scutellatus* species complex (Figure 2a–e), characterised by the squarely protruding apex of the male aedeagus [14]. Two described species not included in the *G. scutellatus* complex (*G. notographus* Boisduval and *G. cinnamomeus* Pascoe) were also collected. Furthermore, two species that have yet to be described were collected and are referred to here as *Gonipterus* sp. n. 6 and *Gonipterus* sp. n. 7. Three species, coded here as *Gonipterus* sp. 8, *Gonipterus* sp. 10 and *Gonipterus* sp. 11 could not be identified at the species level and further taxonomic studies are needed. A variety (synonym) of *G. inconspicuus* Lea was identified but needs taxonomic revision, here referred to as *Gonipterus* sp. 12 (Natalia M. de Souza pers. comm).

Figure 2. Aedeagal sclerites of the 13 *Gonipterus* species identified. Aedeagi of the *G. scutellatus* cryptic species complex (**a**–**e**). *Gonipterus* species phylogenetically grouped with the *G. scutellatus* species complex (**f**–**j**). *Gonipterus* species not part of the *G. scutellatus* cryptic species complex (**k**–**m**). (**a**) *Gonipterus* sp. n. 1; (**b**) *Gonipterus* sp. n. 2; (**c**) *Gonipterus* sp. n. 3; (**d**) *Gonipterus* sp. n. 4; (**e**) *G. pulverulentus*; (**f**) *Gonipterus* sp. n. 6; (**g**) *Gonipterus* sp. 12; (**h**) *Gonipterus* sp. n. 7; (**i**) *Gonipterus* sp. 10; (**j**) *Gonipterus* sp. 11; (**k**) *Gonipterus* sp. 8; (**l**) *G. notographus*; and (**m**) *G. cinnamomeus*.

CO1 sequences were obtained for 108 specimens of which 14 sequences were excluded due to the presence of stop codons, indicating that a pseudogene had been amplified which could lead to the overestimation of genetic diversity. The analyses yielded 17 moderately to strongly supported clades (Figure 3). Of these, 16 represented species of *Gonipterus* and one represented the outgroup, *Haplonyx* sp. Twelve of the 16 *Gonipterus* clades accommodated samples collected during this study. These 12 lineages were supported by the identifications based on male genitalia. No sequences were obtained for the *G. pulverulentus* samples collected. Thus, *G. pulverulentus*, *G. platensis*, *G. scutellatus*, and *G. balteatus* Pascoe in the neighbour joining tree were only represented by GenBank samples. The majority of the species did not show phylogenetic grouping based on the geographic location of different populations. This was with the exception of *Gonipterus* sp. n. 1, where the Australian mainland populations were separated from the TAS populations, and *Gonipterus* sp. 10, where the VIC and ACT populations were separated from each other.

Figure 3. Neighbour joining tree of Cytochrome Oxidase 1 (CO1) gene sequences showing the phylogenetic relationship of *G. scutellatus* species complex and four closely related species. Twelve clades of *Gonipterus* can be distinguished. Numbers below nodes indicate the similarity probability (numbers below 30% not shown). Undescribed species of *Gonipterus* is coded as sp. n. 1–7, unidentified species are coded as *Gonipterus* sp. 8, 10 and 11. *Haplonyx* sp. is the outgroup. Geographical location of samples indicated as well as collection number, GenBank accession numbers are used to indicate reference sequences obtained from GenBank. Branches (samples) within a species clade are collapsed if they are from the same Australian state, only GenBank samples or did not show variation within a species clade.

The distribution of *Gonipterus* spp. differed between the regions surveyed (Figure 4). *Gonipterus* sp. n. 1 was collected from south-eastern VIC, NSW, ACT and TAS and *Gonipterus* sp. n. 2 from all the regions where samples were collected on the Australian mainland. *Gonipterus* sp. n. 3 was collected from SA, VIC and ACT. *Gonipterus* sp. 10 was collected from VIC and ACT. *G. notographus* and *Gonipterus* sp. 11 were only found in VIC. *Gonipterus* sp. n. 4, *Gonipterus* sp. n. 6, *Gonipterus* sp. n. 7, *Gonipterus* sp. 8, *Gonipterus*. sp. 12 and *G. cinnamomeus* were only collected in QLD. *G. pulverulentus* was collected from TAS.
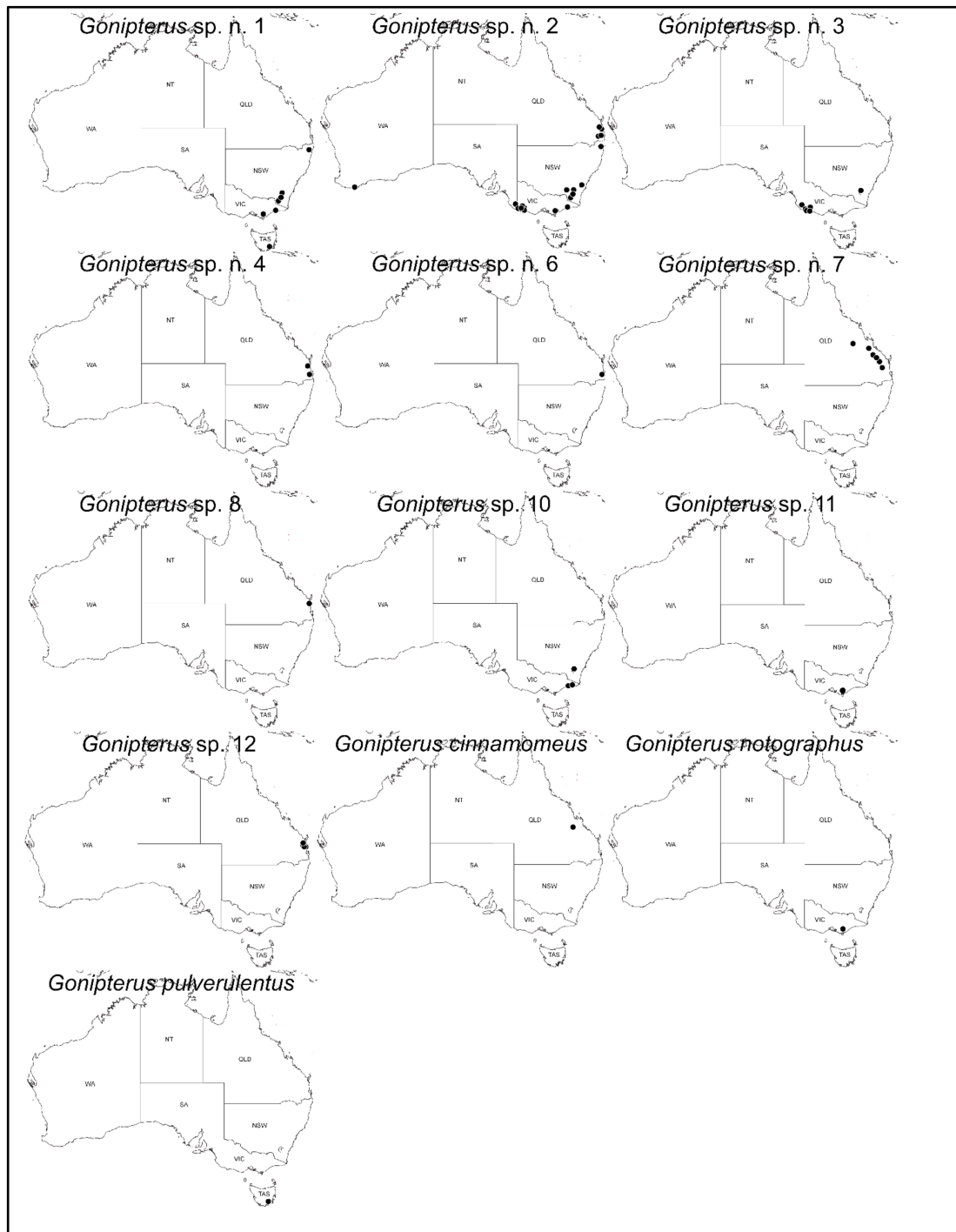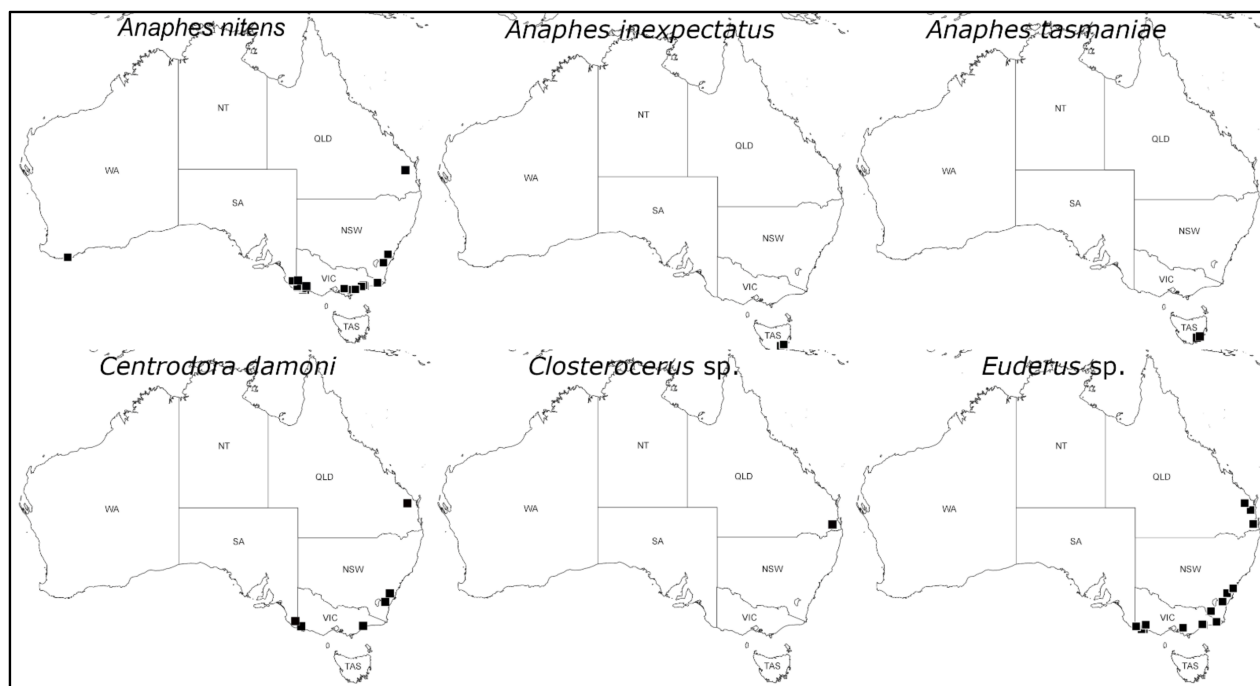

Figure 4. Geographical distribution of the previously recognized *Gonipterus* sp. n. 1–4, the undescribed 6 and 7, unknown *Gonipterus* sp. 8, 10, 11, *Gonipterus* sp. 12 (variety of *G. inconspicuus*), *G. cinnamomeus, G. notographus,* and *G. pulverulentus* in Australia.

3.2. Identification and Distribution of Gonipterus Egg Parasitoids

Five egg parasitoids were identified, including *Anaphes nitens*, *A. tasmaniae*, *A. inexpectatus*, *Centrodora damoni* and *Euderus* sp. (Figure 5). All are known parasitoids of *Gonipterus*. *Anaphes nitens*, *C. damoni* and *Euderus* sp. were collected from all of the collecting regions on the Australian mainland other than WA where only *A. nitens* was collected. *Anaphes tasmaniae* and *A. inexpectatus* were only collected in Tasmania.

Figure 5. Geographical distribution of *Gonipterus* egg parasitoid species, *Anaphes nitens*, *Anaphes inexpectatus*, *Anaphes tasmaniae*, *Centrodora damoni*, *Closterocerus* sp. and *Euderus* sp. in south-eastern Australia.

An additional Hymenopteran species emerged from egg capsule samples collected from south-east QLD (Figure 5). Identification at species level for this insect was not possible because only two individuals were collected, but they appeared to be species of *Closterocerus* Westwood (Hymenoptera: Eulophidae).

3.3. Gonipterus Host Plant Association

The association of *Gonipterus* spp. with *Eucalyptus* spp. differed between the regions surveyed (Table 2). *Gonipterus* sp. n. 1 was collected from *Eucalyptus conspicua*, *E. cinerea* subsp. *cinerea*, *E. crenulata*, *E. globulus* and *E. morrisbyi*. *Gonipterus* sp. n. 2 was collected from the widest range of *Eucalyptus* spp., which includes commercially planted species such as *E. globulus* and *E. dunnii*. *Gonipterus* sp. n. 3 was collected from *E. globulus* and *E. viminalis*. *Gonipterus* sp. n. 4 was collected from *E. propinqua* and *E. microcorys*. *Gonipterus notographus* was collected from *E. radiata* subsp. *radiata* and *Gonipterus* sp. 11 from *E. globulus*. *Gonipterus* sp. n. 7 and *G. cinnamomeus* was collected from *E. melanophloia* and *Gonipterus* sp. 12 from *E. propinqua*. The host association of the *Gonipterus* species is not comprehensive, because many of the samples were collected from unidentified juvenile trees. In total, fifteen species of *Eucalyptus* were identified (Table 2), thirteen in the subgenus Symphyomyrtus and the remaining two species in the subgenera *Eucalyptus* and Alveolata [44].

Table 2. *Eucalyptus* species and their associated *Gonipterus* species. Classification of *Eucalyptus* species according to [44].

Gonipterus Species	*Eucalyptus* Species	Section	Subgenus
Gonipterus sp. n. 1	*Eucalyptus conspicua* *	Maidenaria	Symphyomyrtus
Gonipterus sp. n. 1	*Eucalyptus cinerea* subsp. *cinerea*	Maidenaria	Symphyomyrtus
Gonipterus sp. n. 1	*Eucalyptus crenulata* *	Maidenaria	Symphyomyrtus
Gonipterus sp. n. 1	*E. morrisbyi* *	Maidenaria	Symphyomyrtus
Gonipterus sp. n. 1, *Gonipterus* sp. n. 2, *Gonipterus* sp. n. 3, *Gonipterus* sp. 11	*Eucalyptus globulus*	Maidenaria	Symphyomyrtus
Gonipterus sp. n. 2	*Eucalyptus longifolia*	Similaris	Symphyomyrtus
Gonipterus sp. n. 2	*E. nicholii*	Maidenaria	Symphyomyrtus
Gonipterus sp. n. 2	*Eucalyptus dunnii*	Maidenaria	Symphyomyrtus
Gonipterus sp. n. 2	*Eucalyptus benthamii*	Maidenaria	Symphyomyrtus
Gonipterus sp. n. 2	*E. scoparia*	Maidenaria	Symphyomyrtus
Gonipterus sp. n. 2, *Gonipterus* sp. n. 3	*E. viminalis*	Maidenaria	Symphyomyrtus
Gonipterus sp. n. 2, *Gonipterus* sp. n. 4, *Gonipterus* sp. n. 6	*E. microcorys*		Alveolata
Gonipterus sp. n. 4, *Gonipterus* sp. 12, *Gonipterus* sp. n. 6	*E. propinqua*	Latoangulatae	Symphyomyrtus
G. cinnamomeus	*E. melanophloia* *	Adnataria	Symphyomyrtus
Gonipterus sp. n. 7			
G. notographus	*E. radiata* subsp. *radiata* *	Aromatica	Eucalyptus

* Host not previously recorded for *Gonipterus* spp.

4. Discussion

This study makes a significant contribution towards a better understanding of the distribution of *Gonipterus* species and their natural enemies on the Australian mainland. This study confirmed the native and introduced distribution range of the *Gonipterus* species known from the *G. scutellatus* species complex, extended the known distribution for two species of *Gonipterus* and provided distribution records of two previously undescribed species. In total, 13 species of *Gonipterus* were collected and identified. Nine species were phylogenetically grouped in the *G. scutellatus* species complex, but only five of these shared the squarely protruding apex of the aedeagus that is characteristic of the complex [14]. Six species of egg parasitoids were identified, five species that infest *Gonipterus* egg capsules and the status as *Gonipterus* parasitoid of the sixth species, *Closterocerus* sp., is unknown (Gerhard Prinsloo, pers. comm.). Species of *Closterocerus* parasitoids are known to parasitize leafminers and gall formers [45], for example, *C. mirabilis* Edwards & La Salle attacks Agromyzidae [46] and *C. chamaeleon* (Girault) attacks *Ophelimus maskelli* [47]. The known distribution range for three of the egg parasitoid species known to infest *Gonipterus* eggs was extended.

In this study, we confirmed that *Gonipterus* sp. n. 1–4, and *G. pulverulentus* belong to the *G. scutellatus* cryptic species complex as determined by Mapondera et al. [14]. Two additional undescribed species, *Gonipterus* sp. n. 6 and *Gonipterus* sp. n. 7 are identified in the *G. scutellatus* cryptic species complex based on current definition by morphological characteristics [14] and DNA barcoding. An additional three species, *Gonipterus*. sp. 12, *Gonipterus* sp. 10 and *Gonipterus* sp. 11 were also phylogenetically grouped within the *G. scutellatus* complex but did not have the squarely protruding apex of the aedeagus that is characteristic of the complex [14]. These species could also be separated based on their cuticular hydrocarbon profiles [48]. A taxonomic review of *Gonipterus* and the *G. scutellatus* complex is needed to describe the previously undescribed species and to determine whether the species without a squarely protruding apex is to be included in the *G. scutellatus* complex. Understanding these species boundaries and distributions of *Gonipterus* is important to understand species' interactions within the native range and to identify potential biological control agents.

The results of the present study confirm the native distribution of *Gonipterus* sp. n. 2–4, *G. pulverulentus* and *G. cinnamomeus* as determined by previous studies [14,26,38,49], and provide for the first time distribution records for *Gonipterus* sp. n. 6 and *Gonipterus* sp.

n. 7. Importantly, they also expand the known distribution of *Gonipterus* sp. n. 1 and *G. notographus* to the Australian mainland, given that they were previously only known to be from Tasmania [14]. The *Gonipterus* sp. n. 1 population in Victoria, New South Wales and Australian Capital Territory was phylogenetically distinct from the Tasmania population, suggesting that the mainland population is not a recent introduction. In contrast, the *G. notographus* population in Victoria was not phylogenetically separated from the Tasmanian population, suggesting it could be a recent introduction. Other recent introductions of *Gonipterus* spp. part of the *G. scutellatus* cryptic species complex have also been recorded outside their native ranges within Australia. *G. platensis* and *Gonipterus* sp. n. 2 are invasive in Western Australia [14] and *Gonipterus* sp. n. 2 have recently been recorded in Tasmania [26].

This study confirms that the distribution of the invasive species differs in the native range, which indicates that natural enemies for the development of biological control programs should be collected from the respective distribution ranges within the native range. No new distribution records were recorded for the three invasive species apart from *Gonipterus* sp. n. 2, which was recorded further north- in south-eastern QLD in the present study than the study by Mapondera et al. [14]. *G. pulverulentus* was not collected on the Australian mainland in the present study despite the species being reported previously in NSW and QLD [14]. Thus, it can be inferred from the distribution records from the present and previous studies of the three invasive species that the biological control agent, *A. nitens*, was imported from the native range of *Gonipterus* sp. n. 2, as both the beetle and parasitoid species are present at Penola, from where *A. nitens* was originally collected [24].

The distribution records of the egg parasitoids emerging from the present study together with previous records [24,29,50,51] indicate that three of the five egg parasitoids of *Gonipterus* are present throughout the entire distribution range of *Gonipterus* species on the Australian mainland. Two species, *A. nitens* and *C. damoni* were previously recorded from the Australian mainland, but with limited distribution [24,29,51]. This study presents the first record of *A. nitens* in Queensland: previously, it was recorded from South Australia, Victoria, New South Wales, Australian Capital Territory, Western Australia and Tasmania [24,50,52]. Our results provide the first record of *C. damoni* in South Australia, Victoria and New South Wales. This species has previously been recorded from Queensland, Australian Capital Territory and Tasmania [26,38,51], *Euderus* sp. was recorded from Victoria, New South Wales and Queensland. Previously, *Euderus* sp. had been identified from Western Australia and Tasmania [26,38,50]. Despite the three species known to be parasitoids of *Gonipterus* spp., being present on the Australia mainland and Tasmania, further collections for the development and introduction of biological control agents should be focused on the native distribution of the respective invasive species and consider the local climatic conditions of the target region in comparison to the native range.

The native distribution range of *A. nitens* and *Gonipterus* sp. n. 2 includes five different states on the Australian mainland. The five states are divided into three major climatic zones, which are further subdivided based on regional rainfall [53]. *Anaphes nitens* used in the biological control program worldwide was originally collected from a single population in Penola, South Australia. The Mediterranean climate of this region is not the same across all the regions where the parasitoid is used as a biological control agent. For example, in South Africa, the climate in the Western Cape region is similar to that in Penola, with winter rainfall [24]. However, *Gonipterus* sp. n. 2 has established across a range of different climatic zones within South Africa, including temperate regions of the Highveld with winter temperatures much lower than that experienced at Penola, and sub-tropical regions of coastal KwaZulu-Natal that is more similar to Queensland, Australia [24]. Sub-optimal temperatures for *A. nitens* may therefore be influencing the high infestations of *Gonipterus* sp. n. 2 observed in these regions of South Africa (authors, pers. obs). The same is true for other regions of the world, such as Portugal, where low parasitism rates are associated with maximum winter temperatures below 10 °C [25]. Further introductions of *A. nitens* populations, or other parasitoid species, from the native range to these invaded regions

may be required, where climate matching between collections in the native range, and the target range is considered.

The three egg parasitoid species, *A. nitens*, *C. damoni* and *Euderus* sp. identified on the Australian mainland occur throughout the known distribution range of *Gonipterus* sp. n. 2. This indicates that they do co-exist, but the nature of their interaction is not known. They are all endoparasitoids which share the same niche. It is thus expected that there will be some form of resource competition or niche separation. Niche separation can occur between species that co-exist by having different host and habitat preferences, temporal separation and varying degrees of adaptation to abiotic conditions [54–56]. In our study, *Anaphes nitens* was the dominant species collected at most sites on the Australian mainland, but this could temporally vary. Various aspects of *A. nitens* biology have been studied [24,57–62], but little is known about its interactions with other parasitoid species within its native range. Exploitation and interference competition occurred between *A. nitens* and *A. inexpectatus* in laboratory studies using *G. platensis*, with lower temperatures favouring *A. inexpectatus* [62]. Factors such as the overall efficiency of the parasitoid species, the extent and form of niche separation, and the type of interaction between parasitoids will be important in understanding the final outcome of multiple species introductions on the *Gonipterus* population [63,64].

The present study identified five new host records not previously known from *Gonipterus* species. These include, *G. notographus* associated with *E. radiata* subsp. *radiata*, *G. cinnamomeus* collected from *E. melanophloia* and *Gonipterus* sp. n. 1 collected from *E. conspicua* and *E. crenulata*. *E. radiata* was previously considered resistant to *Gonipterus* attack [24], although these records were from South Africa, where only *Gonipterus* sp. n. 2 is present. Garcia et al. [26] also determined that the host associations for *G. notographus* and *Gonipterus* sp. n. 1 is different from the other species of *Gonipterus* present in Tasmania. Host plant records for the *G. scutellatus* cryptic species have been inconsistent in the past due to the three invasive species being identified as *G. scutellatus* [28]. The present study contributes to a better understanding of host association for ten of the *Gonipterus* species and 15 *Eucalyptus* host species present on the Australian mainland. Understanding host range and niche separation is valuable for planning future studies and more targeted approaches to collect biological control agents in the native range.

The number of *Eucalyptus* hosts identified was limited as most of the collections were from juvenile trees which are difficult to identify due to the absence of flowers and seeds [44]. Future studies could focus on the use of barcoding techniques to identify juvenile *Eucalyptus* trees. The study focused on the Australian mainland and did not include a big sampling effort in Tasmania because Valente et al. [52] and Garcia et al. [26] had recently extensively sampled *Gonipterus* species and their parasitoids in that state. Two of the species detected in their surveys but not in ours were *G. platensis* and *G. scutellatus* [14]. In this study, two collection trips were made at the end and beginning of summer in two consecutive years. Additional surveys in Queensland were undertaken by Souza et al. [65]. A temporal sampling strategy focused on specific regions, climatically matched with the target release area, will contribute to detecting the presence of *Gonipterus* and parasitoid species that were not abundant at the time collections were performed in the present study. This study and the recent surveys undertaken in the native distribution range of the *G. scutellatus* species complex significantly contribute to our understanding of *Gonipterus* biotic interactions in the native and introduced range in Australia and contribute to the development of more robust biological control programs for *Gonipterus* species in the introduced range.

5. Conclusions

This study is the first comprehensive survey of *Gonipterus* species and their parasitoids on the Australian mainland. This study contributes to our understanding of the distribution of *Gonipterus* species in the native range, host relationships and natural enemies. This is important information to underpin the further development of biological control, un-

derstanding native-range interactions, and hasten identification in the event of additional *Gonipterus* invasions.

The results also highlight taxonomic uncertainties that present challenges for the development of biological control agents of *Gonipterus* spp. and in defining species boundaries in the genus. Obtaining clarity regarding the taxonomy of *Gonipterus* spp. will also play an important role in biosecurity. Closely related *Gonipterus* species feeding on commercially relevant *Eucalyptus* species, such as *Gonipterus* sp. 11 collected from *E. globulus* plantations, should be considered by biosecurity practitioners as there is considerable risk that these species escape the borders of Australia and become invasive pests in countries where *Eucalyptus* is commercially produced.

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